FEBS Letters 584 (2010) 555–561

FEBS Letters

journal homepage: www.FEBSLetters.org

# Sialic acids acquired by *Pseudomonas aeruginosa* are involved in reduced complement deposition and siglec mediated host-cell recognition

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ARTICLE INFO

*Article history:*
Received 22 September 2009
Revised 21 November 2009
Accepted 24 November 2009
Available online 27 November 2009

Edited by Sandro Sonnino

*Keywords:*
Complement
Sialic acid
Siglec specific recognition
Visceral leishmaniasis
9-O-acetyl sialic acid
*Pseudomonas aeruginosa*

ABSTRACT

**The opportunism of *Pseudomonas aeruginosa* (PA) in immunocompromised hosts prompted us to explore the potential role of sialic acids (Sia) in this phenomenon. Culture of PA in the presence of exogenous Sia resulted in linkage-specific incorporation of Sia which was associated with decreased complement deposition on the bacteria. Sia acquired by PA mediated enhanced binding of bacteria to recombinant-CHO cells expressing human siglec-7 or siglec-9, as well as to human NK-cells and monocytes naturally expressing these siglecs. Therefore, Sia may be acquired by PA in the host and contribute to bacterial pathogenicity and host-cell interactions via reduction of complement deposition and siglec-dependent recognition.**



## 1. Introduction

*Pseudomonas aeruginosa* (PA) is a Gram-negative opportunistic bacterium, which often infects immunocompromised hosts [1]. The establishment of successful bacterial infection requires protection from the host's plasma components and the association with host cells through appropriate receptor–ligand interactions. Lipopolysaccharides and capsular antigens of Gram-negative bacteria play important roles in maintaining structural integrity and determining the host responses via pattern recognition receptors [2].

We recently identified a sialoglycosylated periplasmic ABC-type phosphate transporter of PA from peripheral blood mononuclear cells (PBMC) of patients with visceral leishmaniasis ($PBMC_{VL}$) [3]. A number of microbial pathogens are able to either synthesize or acquire Sia from the environment as a form of molecular mimicry (Supplementary Fig. S1, [2]). Acquired sialic acid (Sia) is likely to be important in pathogenicity, e.g. via Factor H-dependent inhibition of complement C3-deposition [4]. Recent findings have also shown that Sia binding immunoglobulin-like lectins (siglecs) expressed on various immune cells can promote cellular recognition of sialylated bacteria [5–7].

The presence of Sia on PA and its potential role in interactions with host receptors has not been studied previously. Here, we report the linkage-specific Sia on PA, possibly adsorbed from culture medium. Sia uptake by PA was positively correlated with the prevention of C3-deposition. Furthermore we demonstrate that PA-associated Sia specifically engage siglecs on host immune cells. To the best of our knowledge, this is the first report of Sia being a potentially important constituent of PA, possibly influencing the persistence and association within the immunosuppressed host via siglecs and other host molecules.

## 2. Methods

### 2.1. Detection of Sia on PA

PA, a wild-type, prototrophic, virulent burn wound isolate were grown on Trypticase soy broth (TSB, DIBCO) or on agar plates in a microaerobic atmosphere and harvested after 16 h of growth. Bacteria live or heat-killed (60 °C for 1 h) were washed with

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doi:10.1016/j.febslet.2009.11.087

phosphate-buffered saline (PBS, 0.02 M, pH 7.2) and counted by the spectrophotometric method and confirmed by pour plate colony counts to get colony forming units (CFU).

PA (live and heat-killed) was labeled with FITC, suspended in assay buffer (20 mM HEPES, 150 mM NaCl, 1% BSA, pH 7.4) and enumerated using a bacterial cytometer, a fluorescent microscope and by flow cytometry (BD-FACS Calibur) [6].

Sia liberated from bacteria ($1 \times 10^{15}$ CFU) was purified and separated on a TLC plate [8]. Purified Sia from bovine submandibular mucin (BSM) and authentic Neu5Ac served as standards.

PA ($9.3 \times 10^9$ CFU) grown in only TSB medium, their washes and membrane fractions or PA ($1 \times 10^9$ CFU) grown with Neu5Ac (0–120 min) were oxidized separately to quantify Sia by fluorimetric acetyl acetone method [9]. Relative fluorescence intensity [excitation$_{410\ nm}$/emission$_{510\ nm}$] of the fluorogen was measured with an F-4010 spectrofluorimeter (Hitachi).

1,2-Diamino-4,5-methylenedioxybenzene (DMB)-derivatized purified Sia (0.02 ml) from PA (before and after saponification), equal volumes of fresh and spent TSB media were analyzed by fluorimetric reverse-phase HPLC [3,8]. Each fraction (2–20 pmol/μl) was analyzed by MALDI-TOF-MS (Applied Biosystem) and by ESI-MS on a Q-TOF micromass spectrometer (Waters Corporation) [3].

### 2.2. Detection of linkage-specific Sia

Membrane fractions were prepared from washed PA ($2.5 \times 10^8$ CFU) and analyzed by DIG-glycan and differentiation kits (Roche Applied Science) using *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra agglutinin* (SNA), *Maackia amurensis agglutinin* (MAA), *Peanut agglutinin* (PNA) and *Datura stramonium* agglutinin (DSA) as per manufacturer's protocol [3,8].

Membrane fractions were coated and incubated with biotinylated (B)-SNA and B-MAA overnight at 4 °C and detected using horseradish peroxidase (HRP)-avidin and read on an ELISA-plate reader. The occurrence of Sia on PA ($2.5 \times 10^6$ CFU/ml) in the absence and presence of exogenous Neu5Ac (0–120 min) was analyzed separately using B-SNA and B-MAA by flow cytometry.

The status of *O*-acetylation of Sia was analyzed by flow cytometry by incubating PA with FITC-Achatinin-H (0–1 μg) [3]. To confirm the specificity, PA was de-sialylated and de-*O*-acetylated with *Arthrobacter ureafaciens* sialidase and 9-*O*-acetyl haemagglutinin esterase separately. The binding of Achatinin-H with PA was also determined using anti-Achatinin-H antibodies by ELISA [3].

In overlay dot blotting, sialylated and de-sialylated PA were air-dried separately onto nitrocellulose membrane ($\sim 2.5 \times 10^6$ CFU/dot), blocked in PBS-3%BSA and overlaid with several soluble human siglec-Fc chimeras [10]. The blots were washed, incubated with B-anti-human Fc for 1 h at 25 °C and the bound siglec-Fc complexes were detected using HRP-avidin and quantified by densitometric analysis using Master Totallab Software, version 1.11.

In a spectrofluorimetric ELISA, protein A (0.5 μg/μl) was coated overnight and incubated with soluble human siglec-7-Fc and siglec-9-Fc chimeras (0.5 μg/μl, 100 μl) [6]. The wells were washed, FITC-PA ($2.5 \times 10^6$ CFU/well) was allowed to adhere for 10 min at 37 °C [6], washed and the residual fluorescence intensity was measured using a CytoFlour$^{II}$ fluorescent plate reader.

### 2.3. Complement deposition assay

Freshly prepared normal human serum (NHS) was incubated with PA ($1 \times 10^9$ CFU) for 30 min on ice [11], centrifuged and the clear supernatant was designated as adsorbed NHS (Ads-NHS). Both NHS and Ads-NHS were used at 1:40 dilution as sources of complement.

C3-deposition was measured using FITC-anti-human C3α-chain MAbs (SIM27-49) [11]. PA ($1 \times 10^7$ CFU) cultured in absence and presence of exogenous Neu5Ac (1 mM) for 0–120 min, washed and immediately incubated separately without or with 40% NHS, Ads-NHS and heat-inactivated serum (HI-NHS) in assay buffer containing $CaCl_2$ (5 mM), $MgCl_2$ (2.5 mM), at 37 °C for 3 min. The reaction was terminated using ice-cold PBS-BSA containing 5.0 mM EDTA, washed, incubated with FITC-anti-C3 (1:50) in PBS–BSA for 30 min at 4 °C, and C3-deposition was determined by flow cytometry. C3-deposition was compared with sialidase-(10 mU/$10^6$ CFU)-treated PA. Additionally, the classical pathway (CP) was blocked using EGTA (10 mM) and $MgCl_2$ (10 mM) and processed.

After termination of complement reaction both untreated and sialidase-treated PA in TSB and TSB containing Sia were washed, diluted and appropriate dilutions were plated on TSB-agar for bactericidal assay. Bacterial counts were presented as CFU.

### 2.4. Association of PA to host cells by adhesion assay

$PBMC_{VL}$ and cells from normal donors ($PBMC_N$) was separated by Ficoll-Paque. Chinese hamster ovary (CHO) cell transfectants expressing full-length forms of human siglec-7 (CHO-siglec-7) and siglec-9(CHO-siglec-9) were grown to 80% confluence with HAM's F12 medium containing 10% fetal calf serum, standard antibiotics; lifted using PBS and EDTA (2 mM) along with control wild-type CHO(WT-CHO) cells [12].

Washed CHO-siglec-7 and CHO-siglec-9 were incubated for 1 h at 4 °C with FITC-PA (10 bacteria/cell). Binding assays using de-sialylated CHO-siglec cells and FITC-PA along with de-sialylated FITC-PA and de-sialylated CHO-siglec cells were performed. PA (sialylated and de-sialylated) with WT-CHO cells and pre-blocked CHO-siglec-7 and CHO-siglec-9 with anti-siglec-7 (7.7A) and anti-siglec-9 (kalli) MAbs [13,14] were performed similarly. In parallel, binding of FITC-PA with $PBMC_N$ and $PBMC_{VL}$ was performed.

Side- and forward-scatter properties were used to distinguish cells from free bacteria [7]. Results were expressed as a bacterial binding index, calculated by multiplying the FITC-positive cells (%) with the mean fluorescence intensity (MFI). Phycoerythrin (PE)-anti-CD3, PE-anti-CD19, PE-anti-CD56, PE-anti-CD14 for T-, B-, NK-cells and monocytes of the lymphocyte gate in PBMC population were checked for FITC-PA positivity and MFI was calculated.

### 2.5. Statistical analysis

Values show the means ± S.D. of triplicate results from single representative experiments. Three independent experiments were carried out for each assay. Student's *t*-test was used to determine the level of statistical significance.

## 3. Results

### 3.1. Presence of Sia

TLC analysis of bacterial hydrolysates demonstrated the presence of Neu5Ac, its glycolyl derivative (Neu5Gc) and 9-*O*-AcSia (Neu5,9Ac$_2$) in PA (Fig. 1a). Their presence was also confirmed by fluorimetric-HPLC, which exhibited three intense peaks in the chromatogram, as compared to those derived from BSM (Fig. 1b). A marked reduction of the peak coinciding with Neu5,9Ac$_2$ after alkali treatment indicated its sensitivity to saponification. MALDI-TOF-MS of free DMB-Sia showed the presence of the molecular ions corresponding to all three Sia (Fig. 1c).

Comparable amounts of Sia were observed on unwashed bacteria (6.7 ± 0.45 μg), extensively washed bacteria (5.5 ± 0.32 μg) and the membrane fraction (5.46 ± 0.22 μg) of PA by fluorimetric quantitation. To exclude Sia, which were weakly associated with the bacterial surface, PA was washed four times (50 ml/wash) with

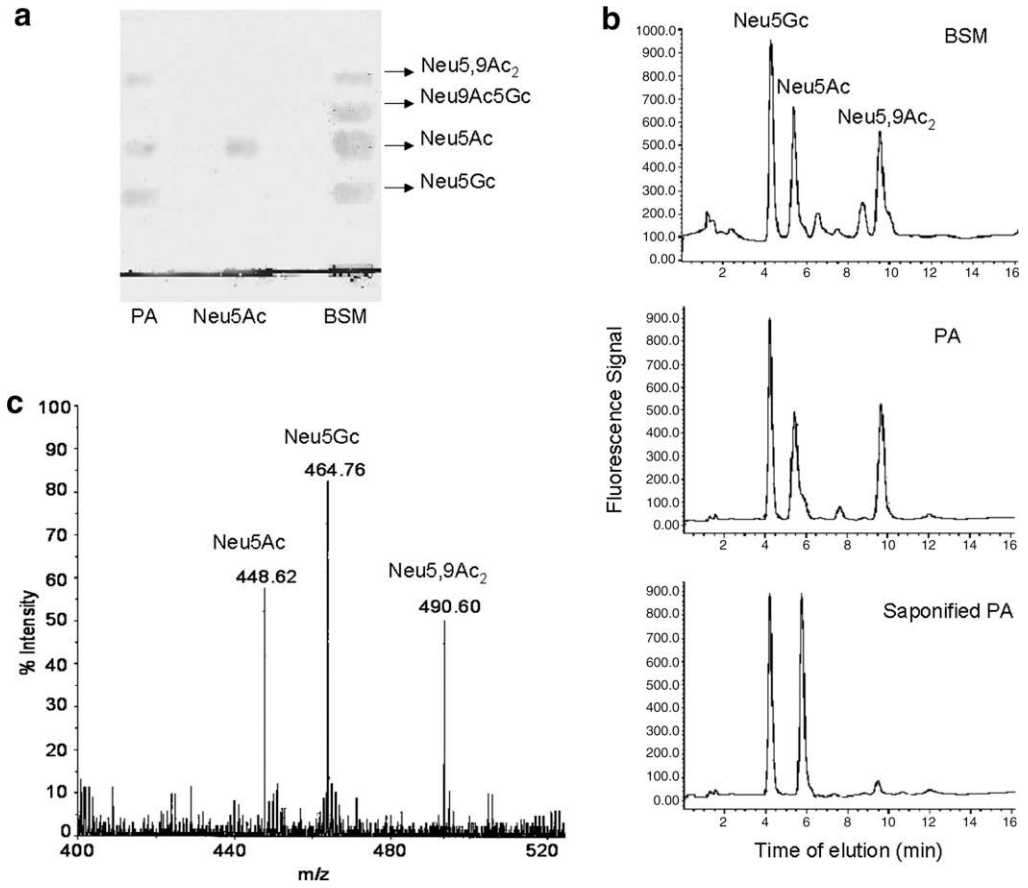



**Fig. 1.** Identification of Sia on PA by TLC (a), fluorimetric-HPLC (b) and MALDI-TOF-MS (c). Purified Sia from BSM and commercially available Neu5Ac served as standards.

PBS. All of the consecutive four washes revealed negligible quantities of Sia (0.21 ± 0.03–0.11 ± 0.02 μg), indicating their strong attachment.

HPLC fractions corresponding to Neu5Ac demonstrated the characteristic molecular ion (*m*/*z* 426, $[M+H]^+$), its sodium adduct (*m*/*z* 448, $[M+Na]^+$) and a characteristic second group ion at *m*/*z* 408, $[M+H-18]^+$ by ESI-MS. The spectra of Neu5Gc displayed characteristic ions *m*/*z* at 424, 442, 464 corresponding to $[M+H-18]^+$, $[M+H]^+$, $[M+Na]^+$, respectively. Similarly, the spectra of $Neu5,9Ac_2$ demonstrated *m*/*z* at 450, 468, 490 corresponding to $[M+H-18]^+$, $[M+H]^+$, $[M+Na]^+$, respectively.

Additionally, each fraction corresponding to Neu5Ac, Neu5Gc and $Neu5,9Ac_2$, yielded the expected signal for the sodium cationized molecular ion having *m*/*z* at 448.7, 464.8 and 490.6, convincingly demonstrating their presence on PA by MALDI-TOF MS.

### 3.2. Presence of linkage-specific 9-O-AcSia

B-SNA and B-MAA specific for the α2,6- and α2,3-linkages exhibited similar dose-dependant binding with both live and heat-killed bacteria, the densitometric scores being 48 555 ± 1102 and 43 355 ± 1042 ($P < 0.001$) (Fig. 2b and c). This lectin binding was lost following sialidase-treatment, the values being 31.13 ± 1.24% to 1.21 ± 0.54% and 30.00 ± 3.26–1.31 ± 0.18%, respectively, as detected by flow cytometry. The binding of PA to the GNA, DSA, and PNA specific for Man, Gal and GlcNAc was much lower.

Densitometric analysis revealed a higher PA-binding with siglec-7-Fc (2436 ± 301) compared to siglec-3-Fc (985 ± 225) (Fig. 2d). The binding was negligible with desialylated PA confirming siglec-specific recognition through Sia.

Although a panel of immobilized soluble siglec-Fc chimeras exhibited complex binding with PA, maximal and minimal binding was observed with siglec-7-Fc (44.20 ± 2.51) and siglec-3-Fc (13.89 ± 2.01), respectively, by spectrofluorimetric ELISA. A marked reduction in the binding of siglec-7-Fc (3.07 ± 1.51) and siglec-3-Fc (3.91 ± 1.81) with desialylated PA provided additional evidence of the presence of Sia on the bacterial surface.

The presence of the $Neu5,9Ac_2$α2-6GalNAc sialoglycotope was confirmed through the dose-dependent binding of Achatinin-H with PA (the OD405 nm being 1.12 ± 0.35, Fig. 2c). De-*O*-acetylation caused a significant reduction of Achatinin-H$^+$PA from 35.52 ± 1.12% to 0.82 ± 0.04%, as detected by flow cytometry. A comparable amount of 9-*O*-AcSia as a percentage of total Sia was detected on intact bacteria (26.37 ± 1.12%) and the membrane fraction (21.42 ± 1.05%) (Fig. 2e).

### 3.3. PA acquire Sia from the culture medium

Fresh TSB medium revealed the presence of Neu5Ac and Neu5Gc (Fig. 3a), which was confirmed by MALDI-TOF-MS (Fig. 3b). TSB medium contained ~5.13 μg/ml of Neu5Ac, whereas spent medium had reduced levels (~4.0 μg/ml) based on fluorimetric estimation. These observations, suggest that TSB provides a source of Sia for the PA surface-associated Sia.

PA ($1 \times 10^9$ CFU) cultured for 120 min in TSB medium displayed only 0.60 ± 0.08 μg of Sia, whereas in the presence of exogenously added Neu5Ac under identical conditions considerably higher levels of Sia were observed (2.75 ± 0.17 μg). A linear increase in binding with B-SNA was observed when PA was cultured in TSB medium containing exogenously added Neu5Ac (0–120 min), indicating enhanced uptake of Sia (Fig. 4a).

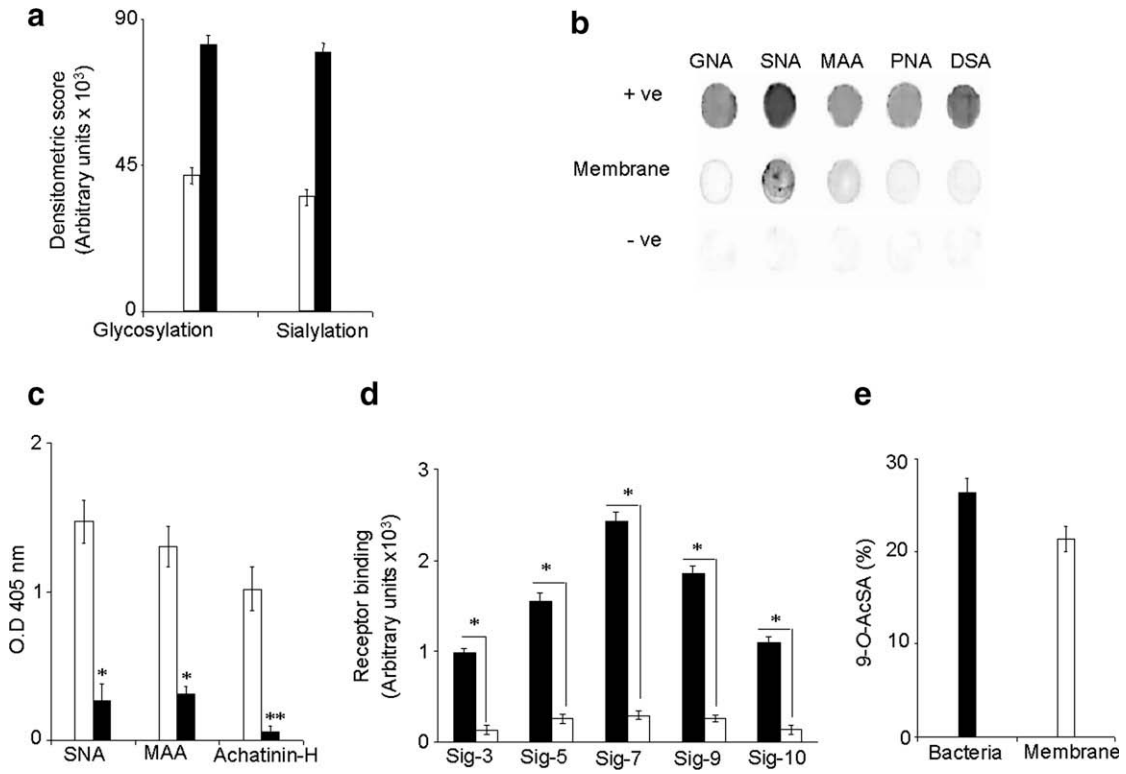



**Fig. 2.** Linkage-specific Sia on *PA*. (a and b) Densitometric scoring of glycosylation and sialylation profiles of membrane (□) by (a) DIG-glycan detection kit as compared to controls (■) and (b) differentiation kits. (c) ELISA. The binding of PA-membrane before (□) and after (■) enzyme treatment with SNA, MAA and Achatinin-H. * indicates $P < 0.005$ for the binding of SNA and MAA with sialidase-treated versus untreated-bacteria, and ** indicates $P < 0.001$ for the binding of Achatinin-H with esterase-treated versus untreated-bacteria. (d) Overlay dot blotting. The binding of sialylated (■) and desialylated (□) PA to soluble human siglec-Fc chimeras. * Indicates $P < 0.001$ for the binding of sialidase-treated versus untreated bacteria. (e) The 9-*O*-AcSia (%) *on PA was* determined by *fluorimetric quantitation*.

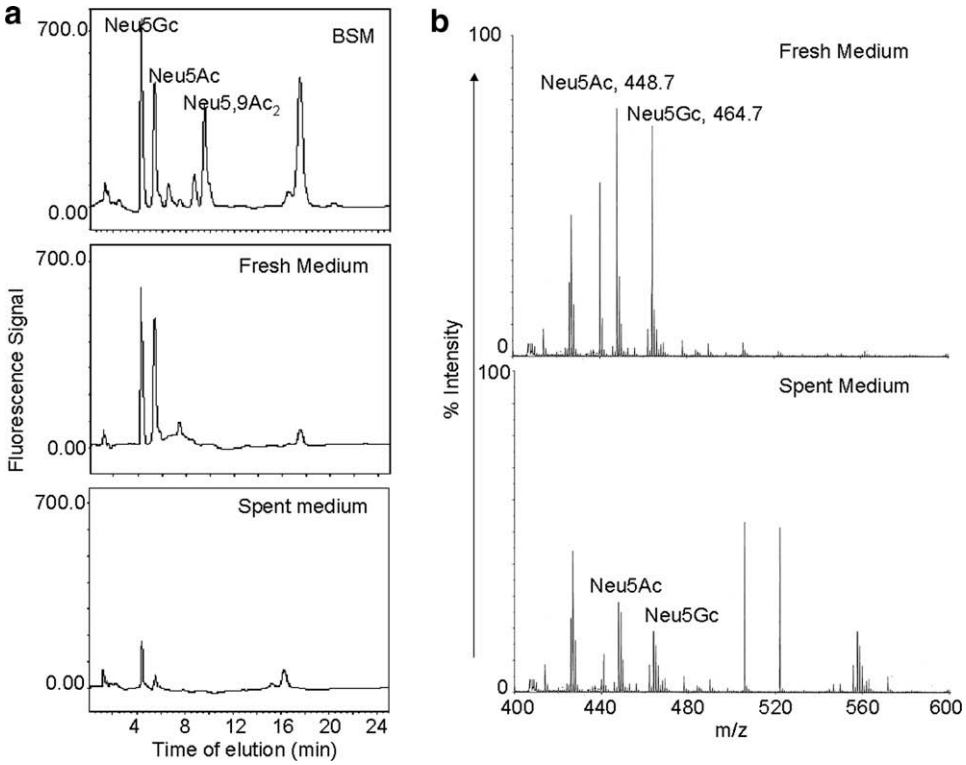
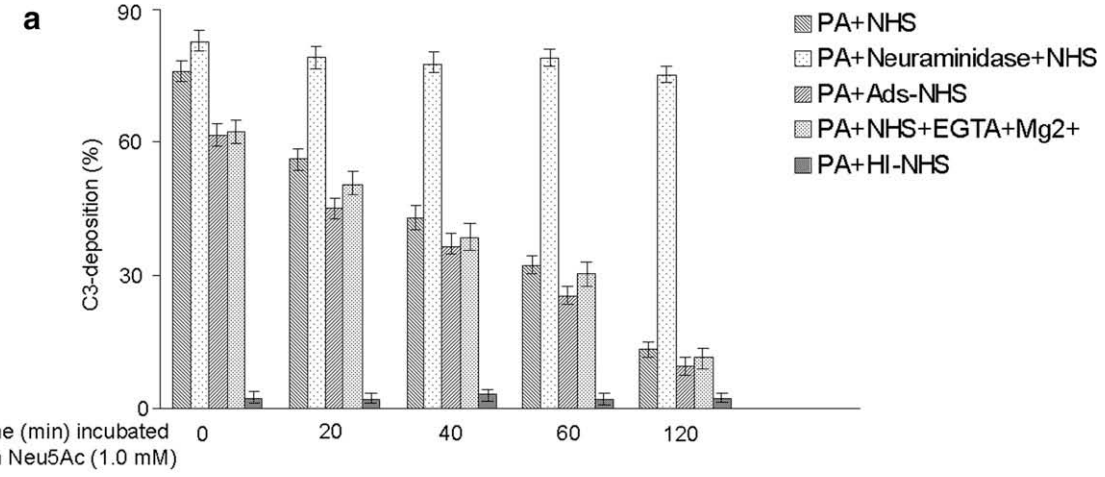



**Fig. 3.** PA acquire Sia from the culture medium as detected by fluorimetric-HPLC (a) and MALDI-TOF-MS (b).

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|---|---|---|---|---|---|
| *SNA$^+$ cells (%) | 1.66±0.86 | 6.77±1.63 | 18.28±3.68 | 33.88±2.16 | 54.58±3.41 |
| **Total SA (ng) | 0.60±0.08 | 0.98±0.12 | 1.20±0.14 | 1.55±0.13 | 2.75±0.17 |
| ***CFU (x10$^4$) | 2.5±0.25 | 4.4±0.18 | 8.6±0.28 | 22.4±2.38 | 84.5±3.12 |
| ****CFU (x10$^3$) After sialidase treatment | 3.3±0.17 | 2.9±0.31 | 3.2±0.22 | 3.1±0.18 | 2.9±0.24 |

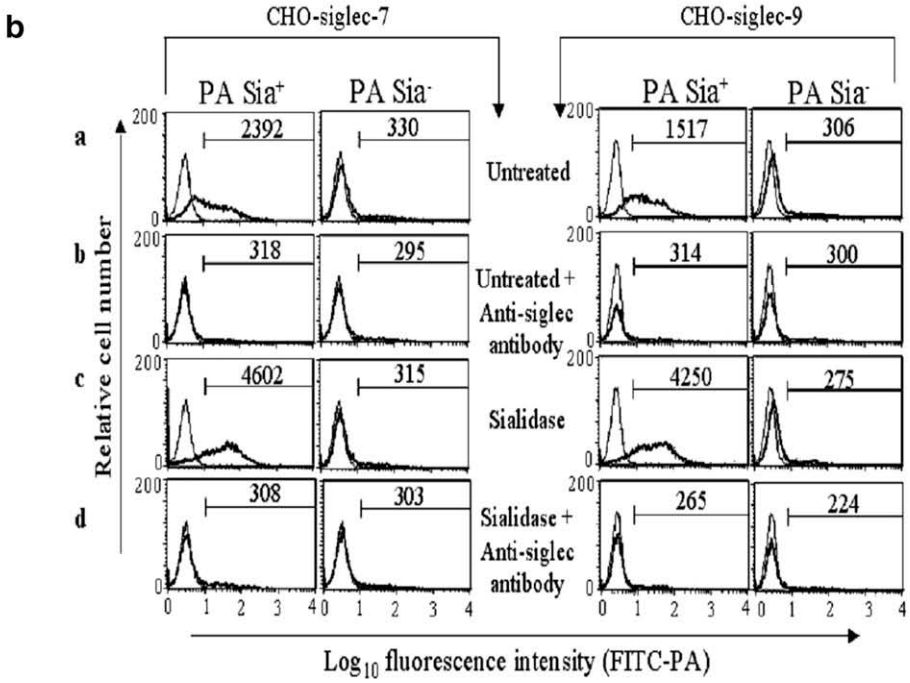


**Fig. 4.** C3-deposition and Sia-siglec recognition. (a) Increased Sia on PA leads to decreased C3-deposition. Sia-positive bacteria* (%SNA$^+$ cells) and acquired Sia was quantitated ** fluorimetrically. The CFU of neuraminidase, *** untreated and **** treated PA was detected by bactericidal assay. (b) FITC-PA binds with CHO-siglec-7,-9. Binding of untreated (PASia$^+$) or neuraminidase-treated (PASia$^-$), (bold-line) is compared with only CHO-cells (thin-line). Binding of PASia$^+$ with (a) CHO-siglecs compared to PASia$^-$; (b) preincubated CHO-siglecs with the respective anti-siglec-7,-9 antibodies; (c) unmasked CHO-siglecs by sialidase; and (d) preincubated sialidase-treated CHO-siglecs with the respective anti-siglecs antibodies.

### *3.4. Sia acquired by PA prevents complement deposition*

In the absence of exogenous Neu5Ac (0 h culture), PA exhibited the highest C3-deposition, with 74.06 ± 3.22% binding with the anti-C3 Mab (Fig. 4a), and yielded $2.5 \pm 0.25 \times 10^4$ CFU. C3-deposition showed a gradual decrease with an increasing exposure time of PA to exogenous Neu5Ac. The binding of PA with anti-C3 after 120 min of exposure was only 12.32 ± 2.75% and had $8.45 \pm 0.31 \times 10^5$ CFU, suggesting that the acquired Sia prevented C3-deposition and as a result resistance to bacterial lysis.

In contrast, sialidase-treated PA, showed significantly increased C3-deposition in the absence (82.65 ± 2.75%) and presence (75.23 ± 1.97%) of exogenous Neu5Ac and decreased CFU of $3.3 \pm 0.17 \times 10^3$ and $2.9 \pm 0.24 \times 10^3$, respectively suggesting the significance of the absorbed Sia in C3-deposition. Ads-NHS showed a similar trend and the absence of C3-deposition with HI-NHS reconfirmed the role of SA.

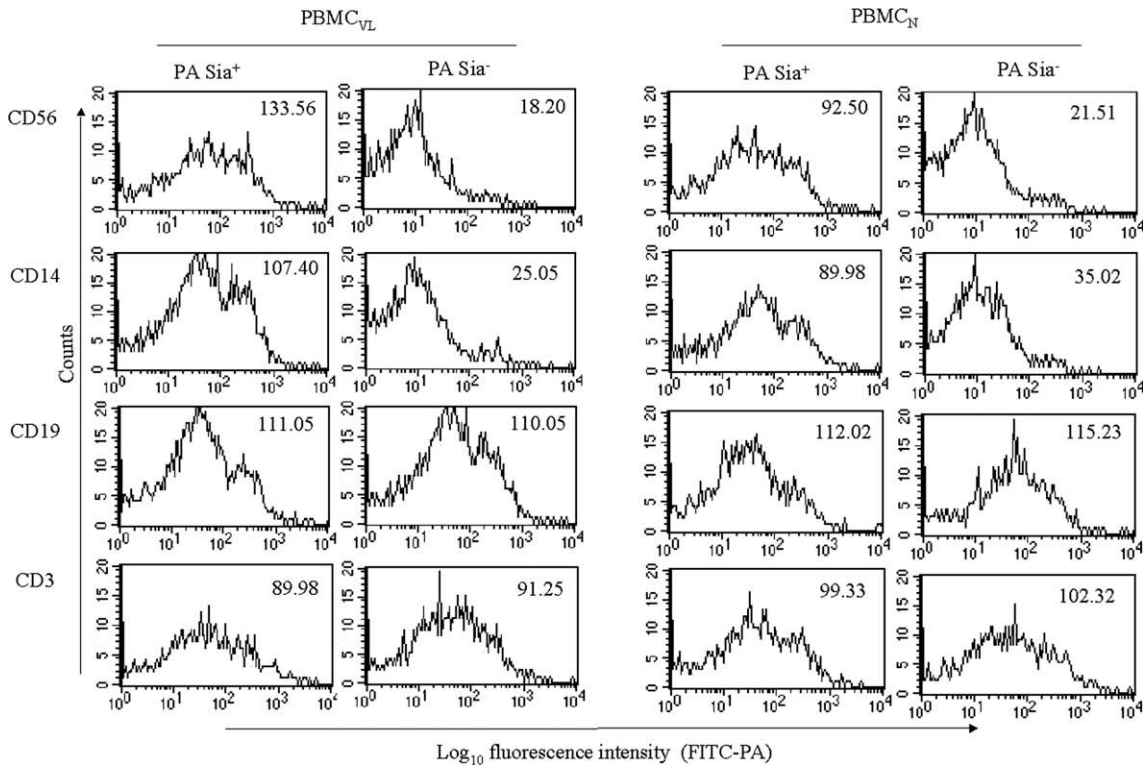


**Fig. 5.** Sia-dependent recognition of NK-cells and monocytes by *PA*. The MFI associated with the specific cells gated for FITC-PA fluorescence.

### *3.5. PA binds in a Sia-siglec-dependent manner*

#### *3.5.1. CHO-siglec cells*

The potent binding of PA with recombinant soluble siglec-7 (Fig. 3d) inspired us to investigate whether cells expressing siglecs could interact directly with PA. The binding of FITC-PA with CHO-siglec-7 was considerably higher than the control CHO cells, indicating a siglec-dependent association (Fig. 4b). Desialylation of CHO-siglec-7,-9 to unmask siglecs from *cis*-interactions [10] consistently resulted in increased overall binding of PA (Fig. 4b). The Sia dependence of bacterial binding was confirmed by a considerable reduction in the binding of desialylated FITC-PA with CHO-siglec-7,-9. In addition, binding was almost abrogated after pre-blocking the cells with anti-siglec-7 and anti-siglec-9 antibodies, indicating siglec-specific recognition.

#### *3.5.2. PBMC$_N$ and PBMC$_{VL}$*

Human siglec-9 is present on monocytes whereas siglec-7 is expressed strongly on natural killer (NK) cells [5]. In flow cytometry-based binding assays, monocytes(CD14$^+$) of PBMC$_{VL}$ evidenced a 4.3-fold reduced binding to sialidase-treated FITC-PA as compared to only 2.5-fold reduction observed with monocytes of PBMC$_N$ (Fig. 5). Thus PA showed sialidase-sensitive binding to both NK(CD56$^+$) cells and monocytes. The PA-binding of T(CD3$^+$)- and B(CD19$^+$)-lymphocyte population of PBMC$_{VL}$ was not reduced following sialidase-treatment representing Sia-independent binding. The binding of FITC-PA with NK-cells and monocytes of PBMC$_N$ was lower than the immune cells of VL, suggesting enhanced cell-specific recognition in the immune-suppressed host.

## 4. Discussion

The present study conclusively establishes the presence of Sia on cultured PA and provides evidence for their time-dependant acquisition from the medium containing exogenously added Neu5Ac. Most significantly, PA-Sia is a potentially important determinant of the protection against complement deposition and may play a role in association with the host cells via siglecs.

Glycosylation in PA has been reported in the form of an *O*-linked glycan in pilin, identical to the lipopolysaccharide *O*-antigen of this bacterium [15]. A cluster of seven pel genes encoding proteins with similarity to the components involved in the polysaccharide biogenesis is found in PA [16]. In general, the regulation of sialylation depends on the balance between sialyltransferase, sialidase, *O*-acetyltransferase and esterase [8]. Bioinformatic searches through the genome of PA indicated the absence of defined sialylation machinery, raising the possibility that the medium could be an alternative source for PA-Sia. Interestingly, PA cultured with exogenous Neu5Ac demonstrated enhanced time-dependant acquisition of Sia. However, whether Sia are transferred wholly or partially remains an area of future research.

Pathogens often protect themselves from the activity of plasma complement-mediated lysis and microbial sialylation has been shown to inhibit complement activation [4,17]. Interestingly, PA cultured for 120 min in non-supplemented TSB medium exhibited a 6-fold higher anti-C3 binding compared to PA cultured in the presence of exogenously added Neu5Ac. This provided evidence for a direct correlation between the Sia levels and the hindrance of C3-deposition on PA, suggesting a way of escaping the plasma defense mechanism.

Another possible way that sialylated pathogens protect themselves from immune responses is to engage inhibitory siglecs expressed on various cells of the innate immune system [5–7]. Most siglecs are masked by *cis*-interactions with Sia present on the same cells but they can be unmasked by desialylation [10]. Here, strong binding to PA-Sia was observed with siglec-7 and -9, both as recombinant proteins and expressed on transfected CHO cells. Selective Sia-dependent binding of PA-Sia to PBMC was observed with sialidase-treated NK-cells and monocytes. As these cells express unmasked forms of siglec-7,-9, respectively [5] they

may play a role in promoting bacterial interactions during natural infections *in vivo*.

Although PA stimulates a complex inflammatory response, the specific contributions of different mediators in the host-defense against this bacterium have yet to be fully characterized. As far as the VL-host is concerned, their associations with immunosuppression provide a convenient gateway for the bacteria. We propose that the Sia of PA contributes to the inhibition of C3-deposition, thus helping them to persist within the host. Acquisition of Sia may also promote association with the immune cells through siglec-dependent recognition. As many siglecs have features of inhibitory receptors [5], this could lead to immunosuppression and hence contribute to bacterial virulence.

## Acknowledgements

This work received financial support from the CSIR (I.I.C.B). P.R.C. is supported by a Wellcome Trust Senior Research Fellowship WT081882MA. We are thankful to Prof. R.D. Cummings, Emory University School of Medicine, Atlanta; Dr. A. Toreno, Instituto de salud Carlos III Majadahonda, Madrid and Dr. R. Vlasak, Applied Bio Technology, Austria, for providing bacterial strains, anti-C3 antibody and *O*-acetyl esterase, respectively.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.11.087.

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